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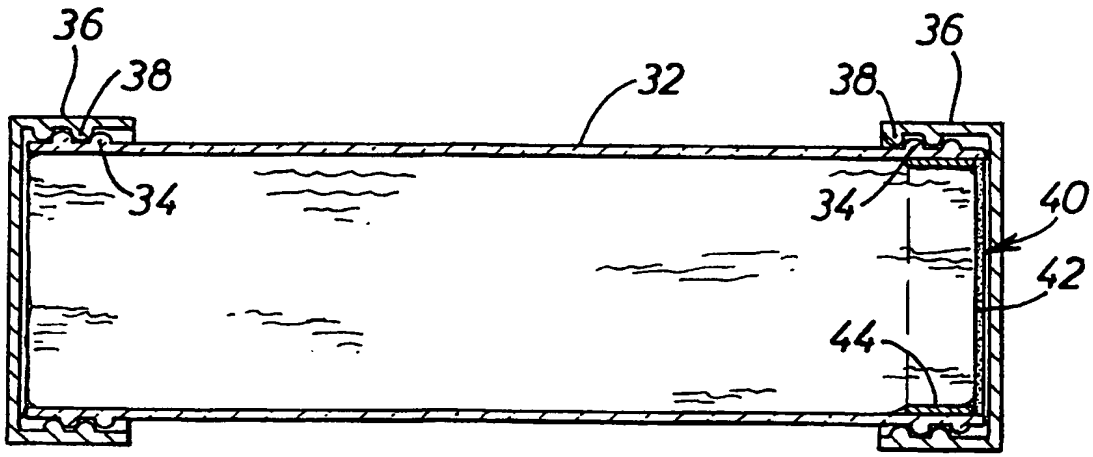

(57) Abstract

Subculturing apparatus (30) comprises a receptacle for supporting a growth medium (5) for culturing of a microorganism. The microorganism is entrained to grow in a predetermined direction, towards an end of the growth medium (50), where a further apparatus (30) can be placed adjacent thereto. The microorganism can then grow into the growth medium of the further apparatus. The method of storing a microorganism for use in microbiological processes is also described, as is a method of fermentation of a stored microorganism for the production of biochemicals such as pharmaceuticals or agrochemicals.

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APPARATUS FOR PRESERVING MICROORGANISMS

The present invention relates to preparing and maintaining cultures of microorganisms, and is particularly applicable to fungi and bacteria.

Microorganisms are essential to many important biotechnical processes including the production of foods, fine chemicals such as vitamins and organic acids, pharmaceuticals, enzymes, agrochemicals and biological control agents. In the pharmaceutical field, microorganisms have yielded drugs which are used for treating infections, disorders of the central nervous system, cardiovascular disease and for suppression of the immune system to prevent rejection following organ transplantation. They hold enormous potential for producing new pharmaceutical compounds.

As a consequence of the economic and academic importance of microorganisms, microbial genetic resource collections have been established to provide cultures of microorganisms for research. The World Data Center for Microorganisms has been assigned the role of characterising, cataloguing and most importantly preserving microorganisms in a pure, viable and

genetically stable condition. Over 500 collections of microorganisms have been registered with the World Data Center.

In industry, where collections can exceed 50,000 microorganisms, and each has the potential to yield a valuable new product or process, it is imperative that microorganism cultures are stored under conditions that maintain genetic stability. Genetic deterioration of a microorganism during storage can result in a reduction or total loss of its biotechnological properties (known or as yet undiscovered). This can result in a significant financial loss to a company.

There are two main approaches to microbial preservation. Firstly, a culture can be maintained on a growth substrate by means of repeated sub-culture onto a new substrate as the growth substrate deteriorates. Secondly, it is possible to create an environment where metabolism of a culture is severely reduced or halted (Smith, D & Onions, AHS, (1994) "The Preservation and Maintenance of Living Fungi", 2nd ed. Wallingford, CAB International).

A method of sub-culturing will now be described with

reference to Figures 1 and 2 of the drawings.

Figure 1 shows a Petri dish 10 which is filled with a layer of agar 12 through which is dispersed a nutrient medium containing sources of carbon, nitrogen, phosphorus, essential vitamins and other elements required for growth. Agar is a natural carbohydrate substance extracted from seaweed. A sample of an organism 14 is inoculated onto the agar at the centre of the Petri dish 10. The Petri dish 10 is then left in a clean environment for a period of 10 days to 2 weeks, or longer if necessary, and maintained at a temperature suitable to promote growth, e.g. 15-25°C. Following that period, the dish will have the appearance as illustrated in Figure 2. As illustrated in Figure 2, a colony of the organism 14 has developed by the growth of filamentous strands along the surface of the agar 12 in all directions from the original sample.

Then, as illustrated in Figure 2, a sub-culture sample 16 can be taken from the growing edge, so as to sample the youngest and most viable part of the filaments. That sub-culture sample can then be inoculated at the centre of a further Petri dish of agar for further culturing. The sample 16 can be taken by means of a sterile scalpel.

The original Petri dish can then be discarded.

It has been recognised that the above method can lead to sampling errors. Microorganisms, and particularly fungi, are inherently genetically variable. For example, as illustrated in Figure 2, whereas the microorganism normally has a green appearance, the zone 18 of the culture identified by chain lines could have genetically segregated so that it has a red appearance. This phenomenon is known as sectoring.

By taking a sample 16 from the Petri dish as illustrated, only genetically segregated red material would be taken from the Petri dish. Therefore, the process of sub-culturing as described above would, under those circumstances, result in the sub-cultured sample having a different overall genetic make-up from the culture from which the sub-culture was selected. It could be that only the green part of the culture exhibited the biological and/or physiological features which might have an advantage suitable for pharmaceutical or agrochemical application. Therefore, by only taking the one sample, the benefit might have been eliminated. This problem was identified in Smith & Onions (1994), referred to above.

Accordingly, a further sample 16' could be taken as illustrated in Figure 2. This second sample 16' would maintain the full genetic composition of the microorganism, which would reduce the problems resulting from genetic segregation. However, some sampling error may remain, since a technologist may not be able to identify all genetic modifications by observation, and so important genetic material may be discarded as a result of the sub-culturing method described above. Moreover, the proportion of materials of different types to be sampled would be a matter for a sampler to identify, which could introduce further errors.

Additionally, it should be emphasised that all microorganism populations are genetically heterogeneous. Consequently, ongoing sub-culture and growth on a synthetic agar medium can act as a selective pressure ensuring that a proportion of the population best suited to those particular conditions of growth become dominant. Desirable properties of microorganisms can be lost as a result. Long term storage of microorganisms by repeated sub-culture is therefore not desirable.

Avoiding repeated sub-culture by covering cultures grown on agar slopes with mineral oil is a traditional method

still widely used. The mineral oil (liquid paraffin) prevents dehydration and slows down metabolism by reducing oxygen availability (Smith & Onions 1994 previously identified; Smith, D & Kolkowski, J (1996) "Preservation and Maintenance of Cultures used in Biotechnology and Industry", San Diego, CA Academic Press). Although fungi have been successfully stored for 40 years using this method, it has a number of serious disadvantages which include retarded growth of the microorganism on retrieval and an increased risk of contamination.

One of the most widely used methods of creating an environment for storing microorganisms in such a way that their metabolic rate is reduced or halted involves the use of cryopreservation at ultra-low temperatures (Smith, D (1993) "Tolerance to freezing and thawing", Tolerance of Fungi, Editor - Jennings, DH pp 145-171 published by Marcel Dekker Inc, New York Smith, D (1998) "The use of cryopreservation in the ex-situ conservation of fungi" - Cryoletters 19, 79-90). Little metabolic activity occurs below -70°C but recrystallisation of ice, which can cause cell damage, can occur above -130°C. Consequently, microorganisms are stored at temperatures below -130°C; in refrigerators (-135°C to -180°C) or in liquid

nitrogen vapour at -196°C. Cellular damage due to ice crystal formation can occur if the freezing and thawing rates are not carefully controlled and the use of cryoprotectant chemicals is important to minimise this damage (Smith (1998) previously referred to; Smith D & Thomas VE (1998) "Cryogenic light microscopy and the development of cooling protocols for the cryopreservation of filamentous fungi", World Journal of Microbiology and Biotechnology, 14, 49-57).

Genetic selection can also be a problem with this method of storage; only a small amount of culture biomass is taken for cryopreservation and only a proportion of that small amount may be viable when the material is thawed.

Cryopreservation is the most expensive in terms of capital equipment required, its running costs and preparation of cultures for storage.

For organisms that sporulate in culture, various methods of drying and freeze drying can be employed. Removal of water reduces cell metabolism and many fungal spores can remain dormant but viable in this way for a number of years. Storage in silica gel is a cheap and effective method for fungi that produce thick walled spores and it

maintains good genetic stability. Storage in sterilised soil is used successfully for some soil fungi but loss of genetic integrity is common and there is a high risk of contamination. Freeze drying involves removing water from frozen cell suspensions by sublimation under reduced pressure (Mellor J.D. "Fundamentals of Freeze Drying", Academic Press 1978). It is a widely used method, but it is unsuitable for non-sporulating fungi, there is often a low percentage viability, genetic damage frequently occurs and it requires expensive equipment.

The time taken for microorganisms to recover from storage where their cellular metabolic activity has been reduced can take three weeks or longer. It is not possible to do anything with the microorganism before the end of this period. In addition to the inconvenience caused, this time delay can add significantly to the costs of biotechnological processes.

There is a need for a simple, cost effective system for the storage and sub-culture of microorganisms which maintains cultures in a viable, metabolically active and genetically stable state.

Therefore, a first aspect of the invention has as its

object the improvement of sampling techniques to maintain reliability of sub-culturing as a method of maintaining a sample of an organism.

Moreover, the use of agar is somewhat undesirable, in that it is a synthetic growth environment which is in some respects different from the nutrient environment which microorganisms would naturally encounter. Whereas agar media are designed to simulate, as closely as possible, the combination of nutrients most amenable to the growth of microorganisms, they remain approximations. The main advantage of agar is that it provides a solid substrate that is not broken down by the microorganism.

Therefore, it is a further object of an aspect of the invention to provide a technique of culturing which makes use of naturally occurring substrates.

The invention provides, in a first aspect, a method of sub-culturing which involves maintenance of microbiological material without selection of specific samples thereof.

The invention provides, in a second aspect, sub-culturing apparatus for presenting a sub-culture across

substantially an entire population of a culture.

The invention provides, in a third aspect, a method of generating a metabolite from an organism maintained according to the first aspect of the invention. The invention also provides, in a further aspect, a method of manufacturing a chemical composition from the metabolite generated in accordance with the third aspect of the invention.

A specific embodiment of the invention will now be described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 is a perspective view of a Petri dish in accordance with an example of an existing technique;

Figure 2 is a plan view of a culture on the Petri dish illustrated in Figure 1;

Figure 3 is a perspective view of a receptacle in accordance with a specific embodiment of the present invention;

Figure 4 is a longitudinal section of the receptacle

illustrated in Figure 3 in an initial condition;

Figure 5 is a perspective view of an insert of the receptacle illustrated in Figure 3;

Figure 6 is a perspective view of an alternative insert to that illustrated in Figure 5;

Figure 7 shows a longitudinal section of an end portion of receptacle in accordance with an alternative and specific embodiment of the invention;

Figure 8 is a perspective view of a receptacle of a further alternative and specific embodiment of the present invention;

Figure 9 is a longitudinal sectional view of the receptacle illustrated in Figure 3 in a first stage of use in accordance with a specific exemplary method;

Figure 10 is a longitudinal sectional view of the receptacle illustrated in Figure 3 in a second stage of use in accordance with a specific exemplary method;

Figure 11 is a longitudinal sectional view of the

receptacle illustrated in Figure 3 in a third stage of use in accordance with a specific exemplary method;

Figure 12 is a longitudinal sectional view of the receptacle illustrated in Figure 3 in a fourth stage of use in accordance with a specific exemplary method;

Figure 13 is a longitudinal sectional view of the receptacle illustrated in Figure 3 in a fifth stage of use in accordance with a specific exemplary method;

Figure 14 is a longitudinal sectional view of the receptacle illustrated in Figure 3 in a sixth stage of use in accordance with a specific exemplary method; and

Figure 15 is a longitudinal sectional view of an arrangement of receptacles as illustrated in Figure 3 for use in accordance with an alternative specific exemplary method.

Referring to Figure 3, a receptacle 30 has a generally hollow cylindrical body 32 which is open at both ends. As shown in Figure 4, each end of the body 32 has an external screw thread 34, and is closed by a cap 36 having a cooperating internal screw thread 38. The caps

36 may be fitted on the body 32 sufficiently tightly that a seal is formed to prevent ingress of microscopic contaminants into the receptacle 30.

The material of the body 32 and the caps 36 is of material which is readily sterilisable for use in biotechnological applications. Moreover, the material is preferably transparent, which allows for observation of the interior of the receptacle 30. A suitable material could be glass, or plastics such as polystyrene, polyethylene, polyamide, polyacrylate. Especially important examples of a suitable material are polycarbonate or polypropylene, which can withstand sterilisation by means of hot water vapour at temperatures up to 121°C.

Each cap 36 extends over its respective end of the body 32 to a depth of not less than 25 mm to ensure that the ends of the body remain sterile when one or both of the caps 36 are removed.

The thickness of the wall of the cylindrical body 32 is 1 mm. Alternative embodiments may have thicker walls, for instance 4 mm, but it is preferable that the wall is sufficiently transparent that the contents of the

receptacle 30 can be observed therethrough. Moreover, the body may be of any other suitable shape other than that of a cylinder.

The internal diameter of the body 32 is 22mm, although this can be varied in alternative embodiments, for example up to 100 mm. Moreover, in the present example, the body 32 is 90 mm in length, but other lengths of body 32 are also envisaged, for instance 50, 150 or 250 mm.

An insert 40, as illustrated in Figure 5, comprises a fine mesh 42, supported on a circular collar 44. The insert 40 is placed at one end of the interior of the body 32 (the right hand end as illustrated in Figure 4). The mesh 42 retains the contents of the receptacle 30 in the event of removal of the cap 36 at that end. The collar 44 is of a size suitable for it to form a tight fit within the body 32, to reduce the risk of the insert 40 accidentally falling out of place. The mesh 42 is sufficiently fine as to prevent egress of any growth medium contained in the receptacle therethrough, but not so fine that a filamentous microorganism is impeded from growing therethrough.

Figure 6 shows an alternative insert 40' having two crosspieces 42' supported on a collar 44'. The collar 44' is identical with the collar 44 illustrated in Figure 5. The crosspieces 42' extend diametrically and mutually perpendicularly across the collar 44'. The crosspieces 42' are operative, in use, to retain any contents of the receptacle 30 to the extent that the cap 36 at that end can be removed and replaced without significant shifting of the medium between the crosspieces 42'. It will be understood that the crosspieces 42' act to impede bulk movement rather than actively preventing it.

The use of an insert 40' with crosspieces 42' as illustrated in Figure 6 is particularly appropriate where the receptacle 30 is used to contain a highly particulate growth medium.

An alternative example of a receptacle 30' including a push-type fitting between a body 32' and a cap 36' is illustrated in Figure 7 of the drawings. In that example, the second embodiment of the insert 40', as illustrated in Figure 6, has been fitted at the end of the body 32'. The end of the body 32' is tapered on its exterior surface, and a corresponding interior tapered surface is formed on the cap 36'. The cap 36' can then

be urged onto the end of the body 32' and, by means of friction and selection of suitable taper angles, the cap 36' can be retained on the body 32', forming a tight seal. Other than this push-fit lid fitting arrangement, alternative arrangements are also envisaged, for example a bayonet fitting, and a push and twist fitting.

An alternative embodiment of the receptacle 30' illustrated in part in Figure 7 is further illustrated in Figure 8. A rectangular membrane 46' is incorporated into the wall of the body 32'. The membrane 46' is of a hydrophobic material, such as polytetrafluoroethylene or polysiloxane, which allows the transfer of gases therethrough, for example oxygen, which is required in many circumstances for the growth of microorganisms. Alternatively, or in addition, the membrane may be located within one or other of the caps 36'. As noted with respect to other components of the receptacle 30', the material selected for the membrane 46' should be suitable for withstanding sterilisation by means of hot water vapour at temperatures up to 121°C.

The membrane extends along the length of the body 32', up to a distance of 25 mm from each end of the body 32'.

With reference to Figures 9 to 14, a specific method of storing a microorganism will now be described.

As illustrated in Figure 9, the receptacle 30 previously described is filled with a suitable growing medium 50. A growing medium should contain assimilable sources of carbon, nitrogen and mineral salts.

Assimilable sources of carbon, nitrogen and minerals may be provided by simple or complex nutrient sources. Preferably, complex nutrient sources are used since they reflect more accurately the natural substrates on which the microorganisms grow. The great variety of nutrients present in complex sources may prevent the unwanted selection of genetic variants existing in a microorganism population which can occur by the placement of the population in an unnatural environment.

Complex sources of carbon, nitrogen and minerals may be provided by clean (not containing chemical residues such as fungicides or other pesticides) grains, cereals and seeds. Examples of such sources are tabulated below:

Cereals and grains	Seeds	Pulses	Agricultural waste	Other
Quinoa	Rye grass	Aduki beans	Ground corn cobs	Wood chips
Maize	Sunflower	Whole lentils	Peanut shells	Wheat bran
Millet	Linseed	Soya	Tea leaves	Oat bran
Rye			Straw (especially from wheat, rye, rice, oat and sorghum)	Other brans including corn, soybean, rye, rice
Wheat				Sawdust (especially hardwood)
Oats				Soil
Rice				Peat moss

Ideally, a medium is formed of a mixture of the above materials, to provide optimum conditions for microorganism storage. Moreover, supplements may be added to the mixture, for example calcium sulphate (which separates individual grains), soy oil, yeast extract or peptone. Peptone is a hydrolysed protein which can originate from animal or plant products.

For example, for basidiomycetes such as *Schizospora paradoxa*, collected and isolated from British woodland, a medium consisting of quinoa is suggested. The use of

the receptacle 30 to store a sample of that microorganism will now be described with reference to Figures 9 to 14 of the drawings.

Quinoa is soaked in boiling water, in the proportion of 1 kg quinoa to 1 litre of water. The mixture is left until it has absorbed all of the water. Then, the receptacle 30 is filled with the soaked mixture to a density of 0.8 g/cm^3. Generally, a density within the range 0.6 - 1.0 g/cm^3 would be acceptable.

Excessive compression of the mixture could inhibit filamentous growth of the microorganism, which could lead to differentiation of the microorganism. Insufficient compression could lead to voids appearing in the growth medium as water and medium is consumed by the microorganism, which would result in unsatisfactory growth of the microorganism. Once the receptacle 30 has been filled with the quinoa medium to the appropriate density and sealed by fitting the cap 36, the whole unit is sterilised by exposure to hot water vapour at 121°C for 40 minutes.

A new microorganism population is inoculated into the left hand end of the growth medium 50 as illustrated in Figure 9, i.e. the end not made inaccessible by the insert 40. Inoculation is effected by aseptically placing a sample 52 taken from an originating population grown in agar, or on grain or other nutrient source, and directly placing that sample 52 into the growth medium 50.

Inoculation could also be effected by injection of a liquid carrying the microorganism into the growth medium 50.

Once inoculation has taken place, the receptacle 30 is placed in conditions that allow optimum growth of the microorganism. These conditions include temperatures ranging from 10°C to 27°C, but ideally 18-25°C for filamentous fungi, in a clean environment such as a specially designed growth cabinet room or incubator so as to minimise risk of contamination from other microorganisms or invertebrate pests such as mites. The incubation conditions can include humidity and light

regulation. A moderately humid environment will reduce the risk of medium in each receptacle from drying out. Sporulation of some microorganisms is induced by diurnal light cycles so light regulation may be advantageous if sporulation of a stored microorganism is required. Figure 10 illustrates the expected appearance of a receptacle 30 after this step has been performed.

Once colonisation of the receptacle 30 by the microorganism has been initiated the receptacle is transferred to storage conditions that reduce growth to a minimum. These conditions can be created in purposefully designed storage cabinets, rooms or incubators to provide clean conditions so as to minimise risk of contamination from other microorganisms or invertebrate pests such as mites. The temperature required to reduce growth should be above freezing and may vary from 4°C to 12°C but ideally 6-10°C. The storage conditions may include humidity and light regulation. Figure 11 shows a receptacle 30 after storage thereof for a period of about six months.

When, as shown in Figure 11, the filamentous growth of the microorganism is nearing the end of the receptacle 30 in which is placed the insert 40, the cap 36 at that end is removed, and a left hand end of a further receptacle 30 is placed in abutment with that end. As shown in Figure 12, in order to retain the receptacles 30 in abutment, a collar 54 having internal thread 56 cooperable with the external threads 34 of the bodies 32 is engaged with those external threads 34. At this stage it should be noted that there may be benefit in making the threads at each end of the bodies 32 of opposite sense, i.e. one being left handed and the other being right handed. In that way, a simple twisting action of the collar 54 is sufficient in one direction to draw the receptacles 30 into close abutment, or in the opposite direction to urge the receptacles 30 apart.

Close abutment of the receptacles 30 is preferred, particularly because the growth medium 50 of the further receptacle 30 should contact the insert 40 of the original receptacle 30. In that way, the filamentous growth of the microorganism may continue uninterrupted.

Once filamentous growth has established itself in the further receptacle 30 as shown in Figure 13, the original receptacle is discarded and a sterile cap 36 is placed at the left hand end of the further receptacle. The further receptacle 30 is then stored in the same way, which will result in a filamentous microorganism as illustrated in Figure 14.

The present invention is particularly applicable to organisms which do not produce a resting state such as spores. For these organisms, such as the basidiomycotina, ascomycotina and other sterile mycelia, the mycelium is the source of inoculum. Therefore, by collecting the youngest part of the colony, as a whole, in a further receptacle, the most vigorous part of the colony, and therefore the most viable part, is maintained.

A further example will now be described of use of the apparatus illustrated in Figures 3 to 14 for the maintenance and sub-culture of a microorganism. *Phlebia deflectens* is a basidiomycete which is commonly found

growing on rotten deciduous wood in British woodland.

In preparation, the sub-culturing apparatus 30 is filled with a growth medium 50 consisting of swollen quinoa grain to a density of 0.8 gm/cm^3. The quinoa grain is swollen by soaking for twelve hours in boiling deionised water in the proportion 1 kg of grain to 1 litre of water. Once the apparatus is filled, it is sterilised by the application of hot water vapour at 121°C for 40 minutes, followed by cooling under sterile conditions.

The spores of *Phlebia deflectens* are collected from fruit bodies in the autumn and germinated on agar containing suitable nutrients. Once a viable colony has been established on the agar, a sample can be taken from the growing edge of the colony using a sterile scalpel. The sample 52 is placed at one end of the sub-culturing receptacle 30 prepared as described above using standard microbiological techniques under sterile conditions. The apparatus 30 is then labelled and stored at 22°C for four weeks. After this time, it has been observed that mycelium grows uniformly through the medium 50 to a total

length of 15 mm and the apparatus 30 can then be transferred to a controlled dark environment at 10°C. After a further four weeks, it has been observed that the mycelium grows a further 5 to 8 mm. This indicates that a drop of temperature from 22°C to 10°C reduces the growth of the mycelium by at least 50%. After 24 weeks in the controlled dark environment, the mycelium is likely to have reached the end of the growth medium 50 and a further receptacle 30 can then be added for further sub-culture.

Phlebia deflectens is a basidiomycete that does not produce any resting stage, such as spores, in laboratory culture. Moreover, its growth and viability are observed to become curtailed when grown and sub-cultured on agar for several months. The method described above using the sub-culturing receptacle 30 maintains the vigour and viability of the *Phlebia deflectens* microorganism, and rapid growth has been observed when a sub-culture of the mycelium is then transferred to a Petri dish containing agar medium for viability testing.

Phlebia deflectens grows at a moderate rate in the sub-culturing receptacle 30. On the other hand, other basidiomycetes and ascomycetes grow at a faster rate than the above example, for example 250 mm in four weeks at 22°C. For such robust and fast growing microorganisms, a cooler storage temperature of 6°C is necessary to reduce growth sufficiently to prevent the microorganism from reaching the end of the growth medium 50, and thus requiring further sub-culture, at intervals of less than six months. Otherwise, the storage method involving the use of the receptacle 30 would become quite labour intensive.

Moreover, since the apparatus may be of relatively simple construction, it provides an ideal solution to the problem of preservation of a local biology, especially in developing countries. In recent times, ecological campaigns here have resulted in countries becoming more aware of their diverse local biology, and efforts have been made for the preservation thereof. The preservation of local microbiology is an integral part of that process.

The illustrated embodiment can be stored easily on racks within an incubator. No sampling takes place and so there is less danger of a reduction in heterogeneity of a microorganism stored in accordance with the invention.

Although the embodiment illustrated in Figures 3 to 14 demonstrates how a single receptacle 30 can be connected to another receptacle 30 on a temporary basis to allow for the propagation of a microorganism through growth medium, the invention also contemplates the arrangement illustrated in Figure 15. In that arrangement, a plurality of receptacles 30 of various lengths are connected together by means of collars 56, each receptacle 30 has an insert 40 as previously described, and the end receptacles are closed by means of caps 36.

A microorganism as shown in Figure 15 can be allowed to grow from one end of the arrangement towards the other, and samples may be taken from the colony of the microorganism by disconnecting the arrangement and removing one or more of the receptacles 30 as required.

Connection and disconnection of receptacles 30 is to be carried out in a sterile manner, for instance in the presence of a sterile airflow, or in the near vicinity of a naked flame, for example a Bunsen burner flame.

The above described apparatus and procedures for sub-culturing and maintaining microorganisms in a genetically stable state can be used to improve the process of culturing microorganisms in a liquid fermentation system either as a surface culture or as a submerged culture or any other type of fermentation for use in a biotechnological process.

Fermentation procedures normally employ one or more growth or seed stages to increase microbial biomass to a level which can be used to inoculate the final production medium designed to yield optimal levels of a desired metabolite. Inoculation levels of biomass to subsequent fermentations is critical to the optimal growth of a microorganism and overall productivity (grams product/unit biomass/unit time) of the process.

A growth stage is typically initiated by the introduction of a small amount of microorganism, which has been cultured on an agar medium, to a 250 ml Erhlenmeyer flask containing 30-50 ml of liquid growth medium. The organism is then cultivated by agitation at a desired temperature (20-40°C) for a period dependent on the growth rate of the organism (range 2-10 days). This culture volume can be increased by a factor of ten at each stage by transferring to ten times the volume of fresh medium and so on.

The seed stage is used to inoculate production medium in which the organism is cultivated to produce the desired product which may be extracted and purified and which may have pharmaceutical, agrochemical or other properties.

A problem encountered with the above described fermentation process is that several seed stages are required to generate sufficient biomass to inoculate the production medium.

The sub-culturing receptacle 30 described above is

advantageous in that it can be used to generate sufficient microbial biomass for direct inoculation of production medium. The inoculum may be prepared in a number of ways prior to inoculating the production medium, for example:

i) The microbial biomass mixed with substrate (from the sub-culturing apparatus) can be used as a direct inoculum.

ii) The microbial biomass mixed with substrate can be gently agitated with an aliquot of production medium or other suitable liquid medium. This suspension is then allowed to settle or is centrifuged at very low speed so that the heavier solid substrate materials are sedimented and removed leaving a suspension of biomass which is used as an inoculum.

iii) The microbial biomass mixed with substrate can be suspended in an aliquot of production medium or other suitable liquid medium and gently blended

under aseptic conditions using a Waring (or similar) blender. This procedure produces a substantially homogeneous suspension of biomass and substrate with a much higher inoculum potential i.e. the gentle blending step releases all the biomass from the substrate and breaks it up to produce more growing points.

In each case the optimal inoculation level will be in the range 1% to 10% weight of biomass combined with substrate (from sub-culturing apparatus) to volume or weight of reproduction medium. An ideal inoculation level is 3%-5%.

The following example describes the use of microbial biomass generated in the sub-culturing apparatus as a direct inoculum for the production of the pharmaceutical compound mevinolin by the organism *Aspergillus terreus* Thom ATCC 20542.

A terreus can be maintained and sub-cultured using the above described receptacle 30 using the grain quinoa as

a growth medium. As described previously, the receptacle 30 is prepared by mixing quinoa with boiling deionised water and leaving the mixture for 12 hours. The swollen grain is then packed into the sub-culturing receptacle 30 to a density of 0.8 g per cm^3. The organism can be maintained in this growth medium 50 at a temperature of 10°C until required for initiating a fermentation process. By raising the temperature to 25°C, the growth rate of the organism can be increased and multiple sub-cultures made onto fresh grain (in separate sub-culturing receptacles) as a means of quickly generating microbial biomass for inoculation purposes.

For fermentation, a liquid medium A is prepared in accordance with the following composition:

	grams/litre
Sheftone N-Z soy peptone (Sheffield Products)	10
Malt extract (Oxoid L39)	21
Glycerol (Sigma-Aldrich)	40
Deionised water	add and make up to 1 litre
Adjust pH to 6.3 using 2N NaOH/2N HCl	

60 ml of the above medium is transferred to a 250 ml Erhlenmeyer flask, stoppered with a polystyrene foam bung and autoclaved at 121°C for 20 minutes. 20g of *A terreus* culture growing on quinoa substrate is then removed from the sub-culturing receptacle 30, mixed with 20 ml of sterile medium A and aseptically blended (up to five 2 second bursts) using a Waring blender. 6 ml of the homogenised inoculum is then aseptically transferred to the Erhlenmeyer flask which is incubated at 25°C under static conditions for a further 15 days.

Mevinolin is assayed in both the broth and methanolic extracts of the separated fungal biomass using established procedures employing high performance liquid chromatography (HPLC). Using this method, average levels of mevinolin in the biomass extracts have been recorded as 414 mg/l, while the average level measured in the cell-free fermentation broth was 224 mg/l.

The recorded levels of mevinolin produced using this procedure are comparable with standard inoculation procedures using separate liquid fermentations (usually

using a different medium composition) to generate inoculum for the production medium. The mevinolin can then be isolated and encapsulated for human consumption in accordance with established procedures.

The foregoing description demonstrates that the illustrated embodiment is capable of being used to maintain an organism, of a filamentous nature, by repeated subculturing without suffering from the effects of genetic segregation. Moreover, contamination can be limited by maintaining sterility of the body and the caps, and by exercising caution when connecting and disconnecting bodies from each other.

A wide range of growth media can be used with the apparatus of the illustrated embodiment. However, it will be appreciated that further growth media, including synthetic growth media, could also be used in conjunction with the apparatus. As synthetic growth media improve, their use may be desirable in terms of cost, reliability and sterility.

By using vessels of different lengths, sub-samples of the entire population can be effected easily. By using a particularly short vessel at a particular point in a chain of vessels, a small section of growth medium can be removed from the chain for further analysis of the organism residing therein. By taking a sample at or near to the growing end of the organism, the most viable biomass can be removed and used in inoculation of further apparatus.

CLAIMS:

1. A process for growing an organism, comprising:

providing a first vessel (30) containing a first body of growth supporting material (50) and causing the organism to grow in said material in said first vessel towards a first location;

providing a second vessel (30) containing a second body of growth supporting material (50); and

permitting said organism to grow from the body of material (50) in the first vessel (30) into the body of material (50) in the second vessel (30) through said first location.

2. A process according to claim 1, comprising connecting said second vessel (30) to said first vessel (30) and permitting said organism to grow into said second body (50) of material while said vessels are connected together.

3. A process according to claim 2 wherein said connecting step is performed in a sterile manner.

4. A process according to claim 2 or claim 3, comprising disconnecting said first vessel (30) from said

second vessel (30) after said organism has begun to grow in said second body of material.

5. A process according to claim 4 wherein said disconnecting step is performed in a sterile manner.

6. A process according to any one of claims 1 to 5 wherein said step of causing said organism to grow comprises causing said organism to grow in said first vessel (30) and into said second vessel (30) in a predetermined growing direction.

7. A process according to claim 6 wherein said first and second vessels (30) are, in the predetermined direction, of different lengths.

8. A process according to claim 7 wherein said second vessel (30) is shorter than said first vessel (30), said process further comprising said step of removing said second vessel, after said step of permitting said organism to grow therein, for sub-sampling thereof.

9. A process according to any one of claims 1 to 8, comprising causing said organism to grow in said second body of material (50) towards a second location therein,

providing a third vessel (30) containing a third body of growth supporting material (50) and permitting said organism to grow from said second body of material (50) into said third body of material (50) through said second location.

10. A process of storing a microorganism comprising the steps of:

providing a growth medium (50);

growing a population of a microorganism on or in the growth medium (50); and

sampling said population for subculture;

characterised in that said step of sampling includes sampling across substantially the whole population of the microorganism.

11. A process of storing a microorganism including the steps of:

providing a growth medium (50); and

causing a microorganism to grow on or in the growth medium; characterised in that said step of causing said organism to grow includes training said microorganism substantially in a predetermined direction.

12. A process in accordance with claim 11 wherein said

step of causing said organism to grow includes causing said organism to grow towards a predetermined location, and said method further comprising sampling at said predetermined location to obtain a sample of said organism across substantially the entire population thereof.

13. A process in accordance with claim 12, wherein said sampling step includes the step of placing a sampling medium (50) adjacent said predetermined location for continuing growth of said microorganism thereon or therein.

14. A process of manufacturing a metabolite comprising the steps of:

storing a microorganism in accordance with the method of any of claims 1 to 13;

extracting a sample of said microorganism;

subjecting said sample to conditions suitable for metabolism; and

extracting metabolite from said sample.

15. A method of preparing a pharmaceutical preparation including the step of isolating a metabolite produced in accordance with claim 14.

16. A pharmaceutical preparation including a metabolite the product of a method in accordance with claim 14.

17. A storage device for use in the process of any of claims 1 to 15 including a housing (30), growth medium (50) within the housing (32), and first and second locations on the growth medium, such that a microorganism can be grown from the first location towards the second location where subculturing of substantially the entire population can be effected.

18. A storage device in accordance with claim 17, wherein the housing (32) is tubular.

19. A storage device in accordance with claim 18, wherein the housing (32) is cylindrical.

20. A storage device in accordance with any one of claims 17 to 19 wherein the housing (32) is of a sterilizable material.

21. A storage device in accordance with any one of claims 17 to 20 wherein said housing (32) has formations (34) at said first and second locations of the growth

medium each formation being suitable to engage with a cooperating formation of another of said storage device (30), for propagation of microorganism therebetween.

22. A storage device in accordance with any one of claims 17 to 21 wherein said housing (32) comprises means (40) for retaining said growth medium.

23. A storage device in accordance with claim 22 wherein said retaining means (40) has at least one aperture defined therein for passage of microorganism therethrough.

24. A storage device in accordance with claim 23 wherein said retaining means (40) comprises at least one retaining member (42') across said vessel.

25. A storage device in accordance with claim 24 wherein said retaining means (40) comprises a reticular member (42) across said vessel.

26. A storage device in accordance with any one of claims 17 to 25 wherein said growth medium (50) comprises a natural foodstuff.

27. A storage device in accordance with claim 26 wherein said foodstuff is a vegetal foodstuff.

28. A storage device in accordance with claim 27 wherein said growth medium (50) comprises a quantity of a cereal.

29. A storage device in accordance with claim 27 or claim 28 wherein said growth medium (50) comprises a quantity of seed.

30. A storage device in accordance with any one of claims 27, 28 or 29 wherein said growth medium (50) comprises a quantity of a pulse.

31. A storage device in accordance with any one of claims 27 to 30 wherein said growth medium (50) comprises an agricultural crop byproduct.

32. A storage device in accordance with claim 31 wherein said agricultural crop byproduct comprises at least one of ground corn cobs, peanut shells, tea leaves and straw.

33. A storage device in accordance with any one of claims 26 to 32 wherein said growth medium (50) comprises at least one of calcium sulphate, soy oil, yeast extract

and peptone.

34. A storage device in accordance with any one of claims 17 to 33 wherein said growth medium (50) is sterile.

35. A storage container for use in the storage device (30) of any one of claims 17 to 35, defining a cavity within which growth medium can be contained, said container (32) comprising first and second access means between which growth medium can extend in use, for growth of an organism between said first and second access means in use.

36. A container in accordance with claim 35 including first and second closure means (30), removably closing said access means in use.

37. A storage device for use in the process of any one of claims 1 to 15 comprising:

growth medium (50) for viably supporting a microorganism;

characterised in that

said storage device includes a facility for presenting said population substantially in its entirety

for subculture.

38. A storage device in accordance with claim 37 and further comprising a receptacle (32) supporting the growth medium.

39. A storage device in accordance with claim 38, wherein the growth medium defines a substantially elongate growing path.

40. A storage device in accordance with claim 38 or claim 39, wherein the receptacle (32) includes attachment means (34) for attachment of said device to further culturing apparatus.

41. A storage device in accordance with claim 40, wherein the attachment means (34) is operable to engage the growth medium with growth medium of a further storage device in accordance with any one of claims 37 to 40.

FIG. 1

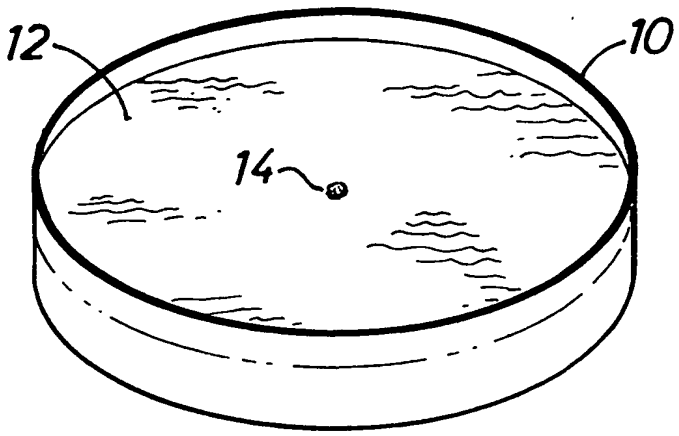


FIG. 2

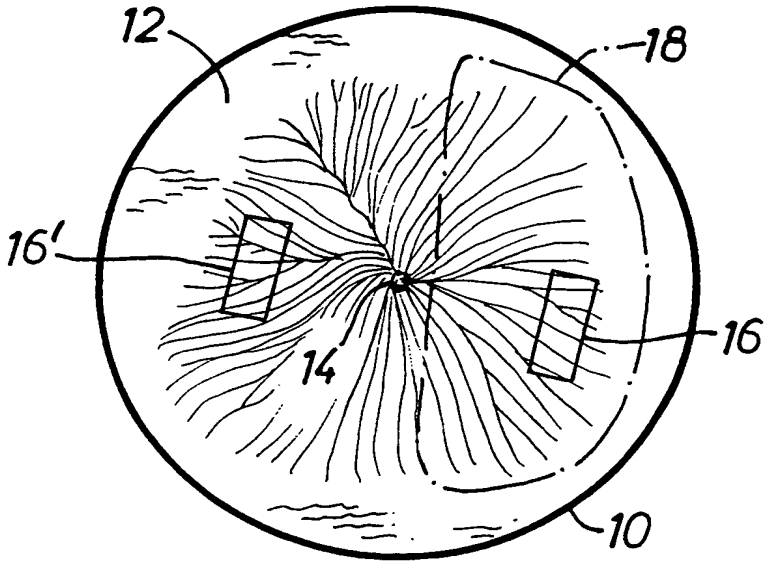


FIG. 3

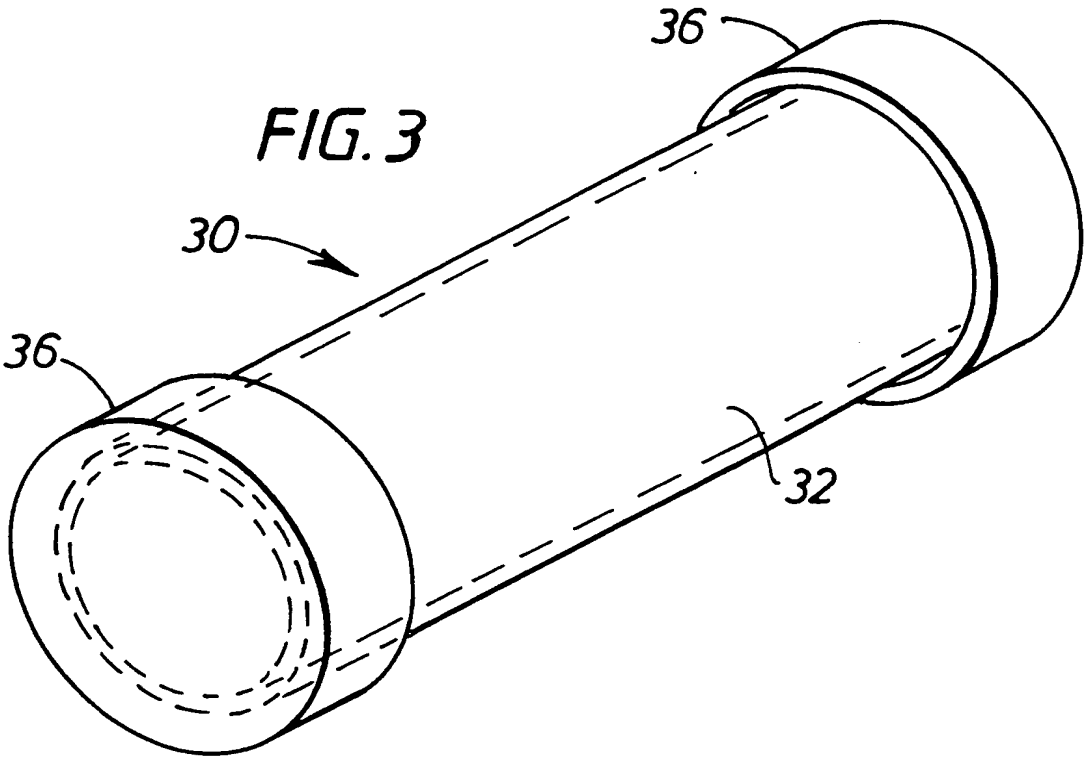


FIG. 4

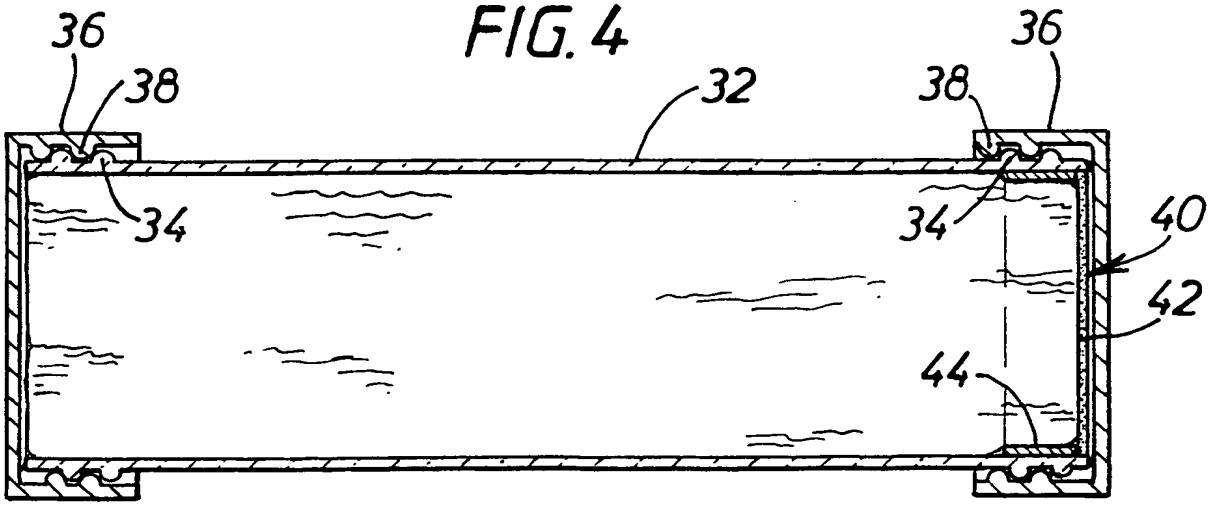


FIG.5

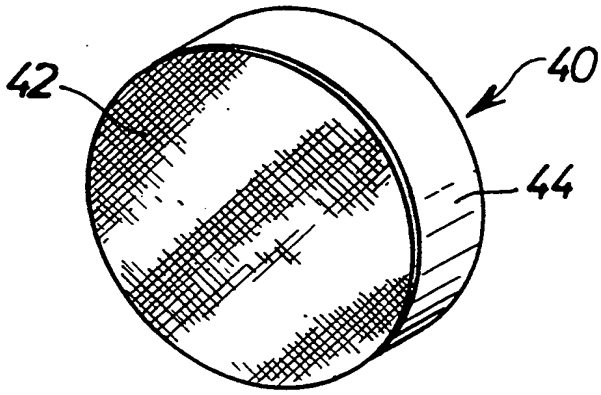
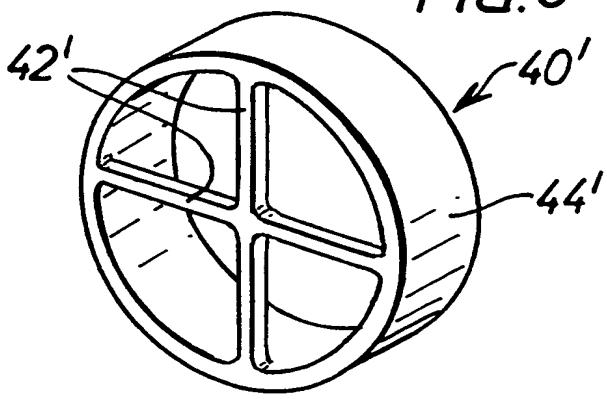


FIG.6



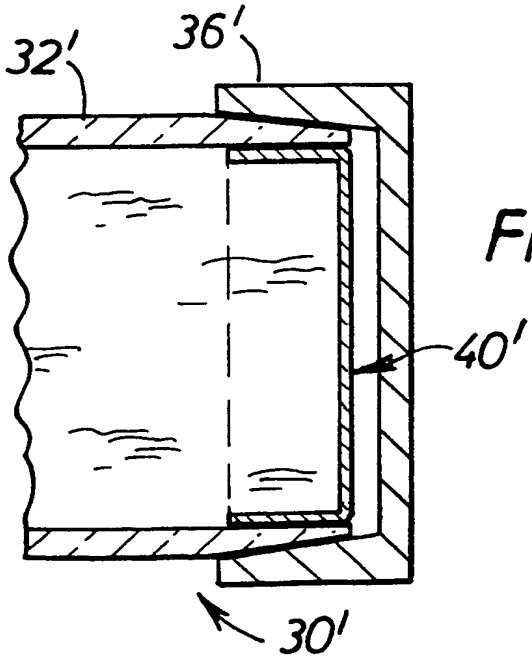


FIG.7

FIG. 8

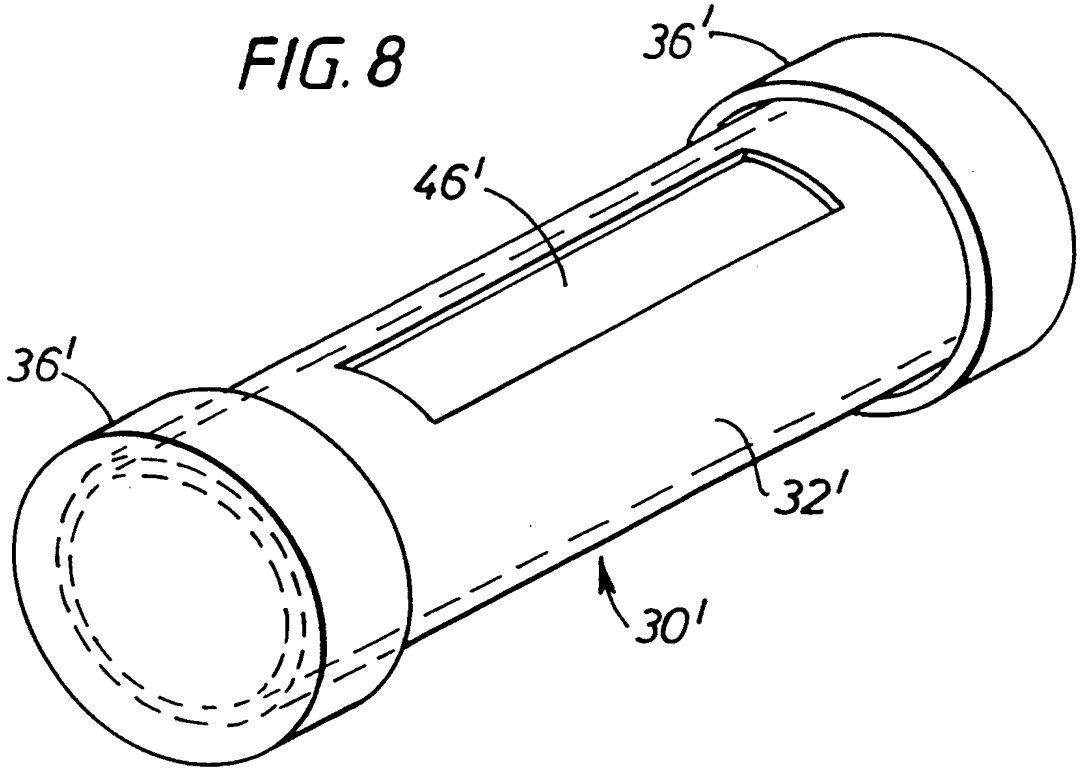


FIG. 9

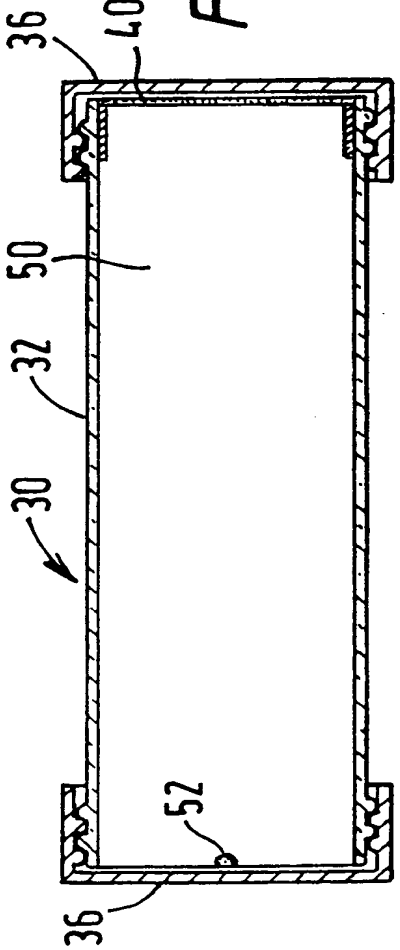


FIG. 10

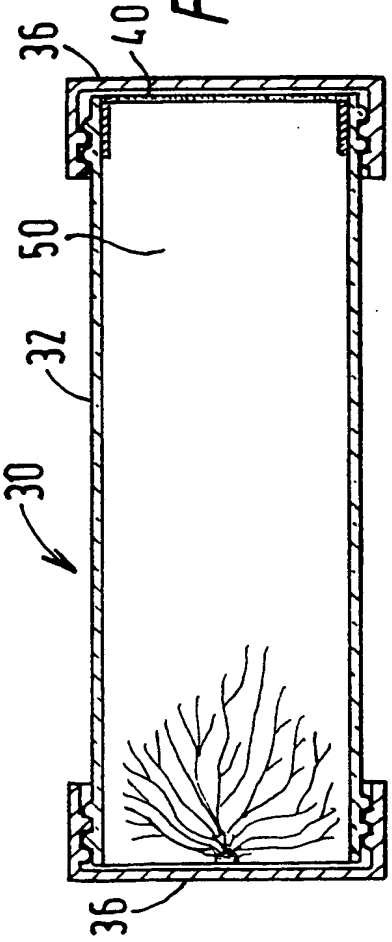
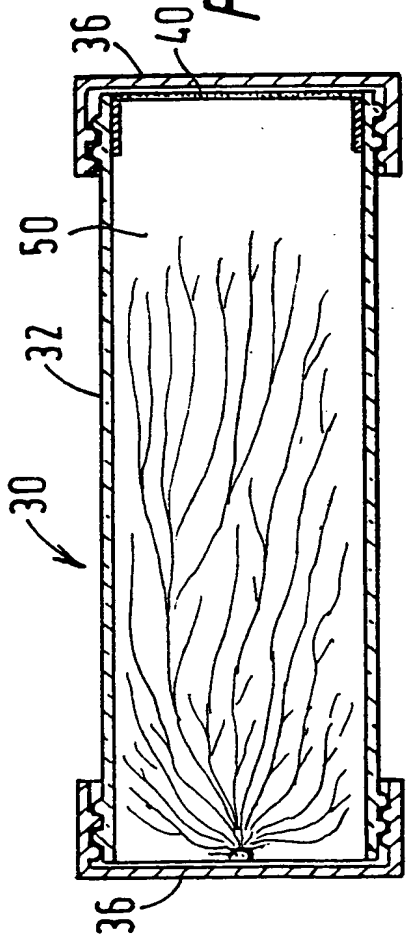


FIG. 11



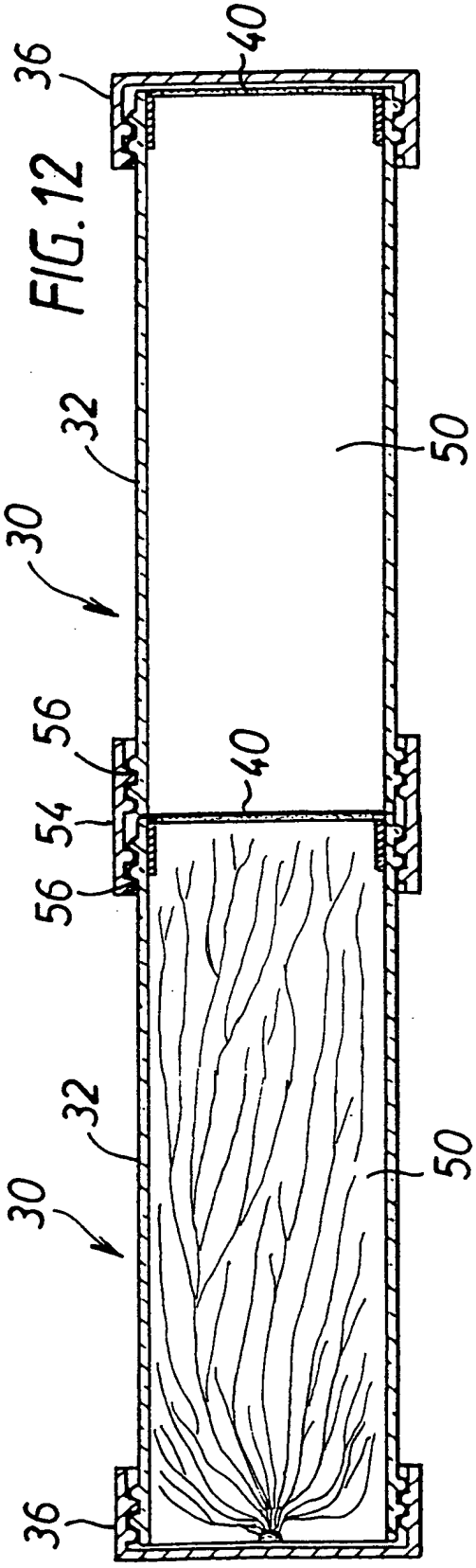


FIG. 12

FIG. 13

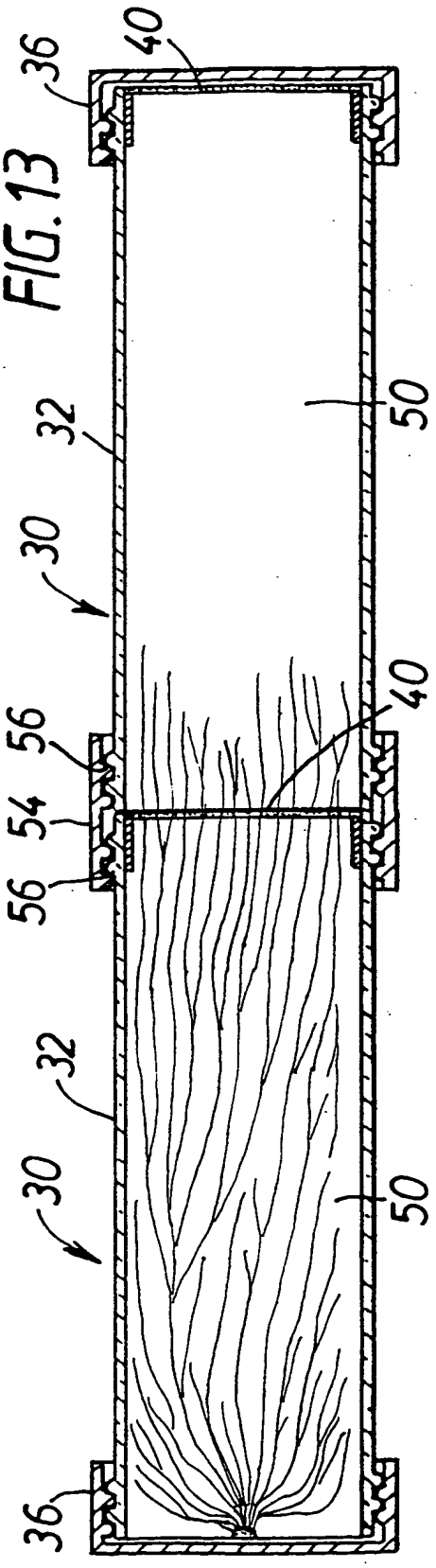
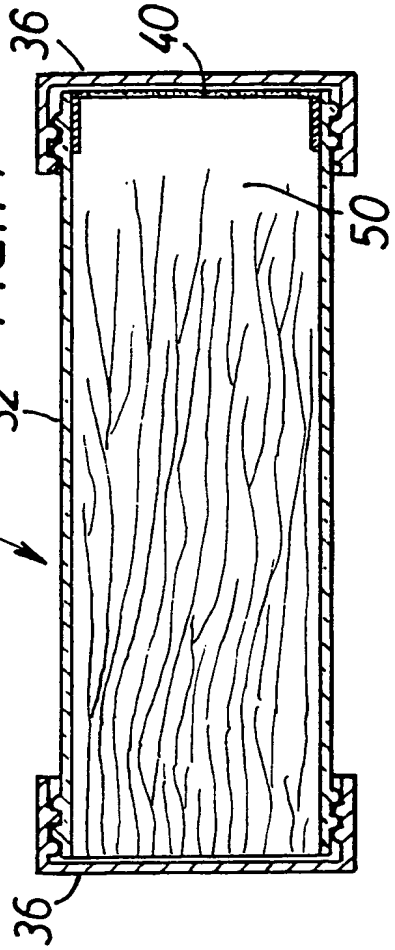


FIG. 14



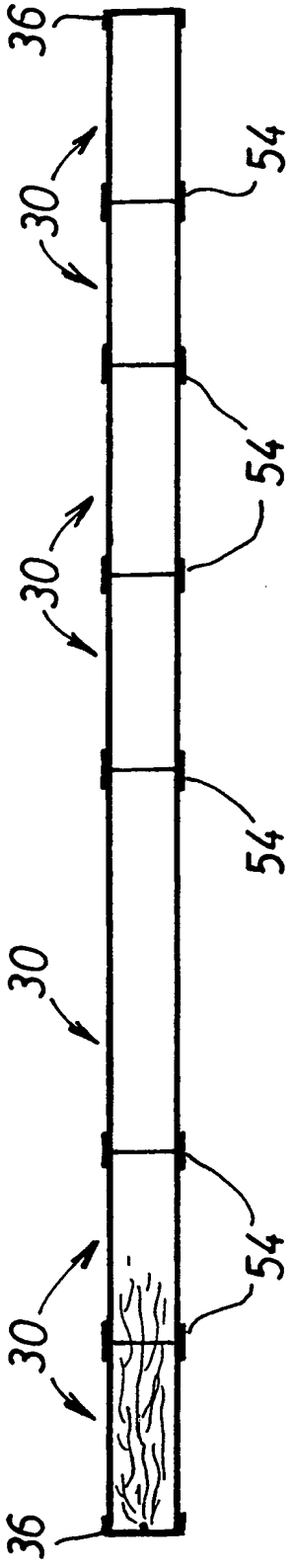


FIG.15

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00378

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12M1/26 C12M1/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI PAJ EPODOC TXTE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 015, no. 154 (C-0825), 18 April 1991 (1991-04-18) & JP 03 030665 A (MITSUBISHI HEAVY IND LTD), 8 February 1991 (1991-02-08) abstract & JP 03 030665 A (MITSUBISHI HEAVY IND LTD) 8 February 1991 (1991-02-08)	1-20,22, 23,26, 33-39
A	FR 403 203 A (LABORATOIRES DE MONTREUX)	
X	EP 0 259 116 A (UNILEVER PLC) 9 March 1988 (1988-03-09)	1,2,6, 9-14, 17-20, 37-39
Y	page 4, line 37 - line 46; claims 1-4,6,7; figures page 10, line 49	15,16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2000	07/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Coucke, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CH 552 063 A (OTTO BIERI) 31 July 1974 (1974-07-31)	1-6, 9-11, 35-37
Y	the whole document	15,16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/00378

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 03030665 A	08-02-1991	NONE	
FR 403203 A		NONE	
EP 259116 A	09-03-1988	AT 84567 T	15-01-1993
		AU 614936 B	19-09-1991
		AU 7769887 A	03-03-1988
		CA 1290273 A	08-10-1991
		DE 3783540 A	25-02-1993
		DE 3783540 T	22-07-1993
		ES 2053550 T	01-08-1994
		US 5403741 A	04-04-1995
		JP 63112975 A	18-05-1988
CH 552063 A	31-07-1974	NONE	

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
5291299		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/ 00378	08/02/2000	08/02/1999
Applicant		
BIODIVERSITY LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 4

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

The Patent Office

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NP10 8QQ

Examiner: 01633 813554
†**E-mail:** james.houlihan@patent.gov.uk
Switchboard: 01633 814000
Fax: 01633 814444

Your Reference: EMR/ACS/5291201
Application No: GB 9902757.5

27 October 1999

Dear Sirs

Patents Act 1977: Search Report under Section 17(6)

In response to the further Patents Form 9/77 filed with your letter of 23 August 1999 and the comments made therein I have conducted a full search in respect of claims 6-15 and 19-21; the search also encompassed claims 16-18, in part, insofar as they are appendant to claims 6-12.

Please find two copies and the search report and a copy of each of the citations.

It is pointed out that claim 11 is apparently anticipated by the action of a laboratory technician taking a sample from a bacterial spinner culture, streaking it on a petri dish and placing the petri dish in a fridge.

Yours faithfully

Dr J Houlihan
Examiner

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†**Use of E-mail:** Please note that under patent law e-mail may be used to file correspondence only.

The Patent Office

INVESTOR IN PEOPLE

Application No:	GB 9902757.5	**Examiner:**	Dr J Houlihan
Claims searched:	6-15 & 19-21 (16-18 in part)	**Date of search:**	27 October 1999

Patents Act 1977
Further Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.Q): C6F (FHB, FX)

Int Cl (Ed.6): C12M 1/00, 1/02, 1/10, 1/14, 1/20, 1/22, 1/24; C12N 1/00, 1/14, 3/00

Other: ONLINE: WPI, EPODOC, PAJ

Documents considered to be relevant:

Category	Identity of document and relevant passage		Relevant to claims
X	WO 86/07376 A	(INST. FRANCAIS DE RES. SCI.) page 2 line 28-page 3 line 26; page 5 lines 5-6	6, 7, 9 & 10

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

The Patent Office

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Examiner: 01633 813554
Switchboard: 01633 814000
Fax: 01633 814444

Your Reference: KB/EMR/ACS/5291201
Application No: GB 9902757.5

28 April 1999

Dear Sirs

Patents Act 1977: Search Report under Section 17(5)

Request for accelerated search

In response to your letter of 9 March 1999 this search has been conducted at the earliest reasonable opportunity.

I enclose two copies of my search report and two copies of the citation.

Plurality of invention

I consider that your application relates to more than one invention as follows:

(i) Claims 1-5 and 16-18 (in part) which relate to a method for culturing an organism wherein the organism in one vessel grows into a medium located in a second vessel.

(ii) Claim 6-11 and 16-21 (in part) which relate to growing an organism so that the entire population is homogenously presented.

(iii) Claims 12-15 and 16-21 (in part) which relate to growing an organism wherein the organism is directed to grow in a pre-determined direction.

None of claims 6, 11, 12 and 19 refer to a second vessel. The only subject matter these claims have in common with claim 1 is a single vessel and medium for culturing an organism and in relation to claims 12 and 19 wherein the organism can be directed to grow in a pre-determined direction. Claim 6 is apparently anticipated by culturing fungi on a

conventional petri dish and claim 11 is similarly anticipated by taking samples therefrom. Claim 12 may be anticipated by a streaked agar plate culture which causes the fungi to grow in a pre-determined direction. Accordingly, claims 6-15, 19-21 and 16-18 (in part) have no unity of invention with claim 1.

You are formally entitled to request further searches for any of the other inventions by filing a separate Patents Form 9/77 for each invention. However, as indicated above, it appears that each of claims 6, 11, 12 and 19 is anticipated by common general knowledge.

Publication

I estimate that, provided you have met all formal requirements, preparations for publication of your application will be completed soon after **4 July 2000**. You will then receive a letter informing you of completion and telling you the publication number and date of publication.

Amendment/withdrawal

If you wish to file amended claims for inclusion with the published application, or to withdraw the application to prevent publication, you must do so before the preparations for publication are completed. **No reminder will be issued.** If you write to the Office less than 3 weeks before the above completion date, please mark your letter prominently: **"URGENT - PUBLICATION IMMINENT".**

Yours faithfully

Dr J Houlihan
Examiner

The Patent Office

INVESTOR IN PEOPLE

Application No: GB 9902757.5 **Examiner:** Dr J Houlihan
Claims searched: 1-5; 16-18 (in part) **Date of search:** 27 April 1999

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.Q): C6F (FHB, FHC1, FHC2)

Int Cl (Ed.6): C12M 1/18, 1/24, 3/00

Other: ONLINE: WPI, EPODOC, PAJ

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	GB 1581832 (UNI. STRATHCLYDE) See figures	

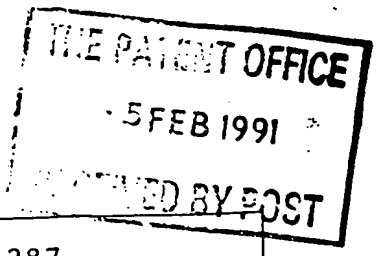
X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

PATENT NO EP(UK) 0223809

TRANSLATION OF EUROPEAN PATENT (UK) UNDER SECTION 77(6) (a)

Date of Publication of the Translation 13-3-91

For official use



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Notes
Please type, or write in dark ink using CAPITAL letters.

A prescribed fee is payable with this form. For details, please contact the Patent Office (telephone 071-829 6910).

Paragraph 1 of Schedule 4 to the Patents Rules 1990 governs the completion and filing of this form.

This form must be filed in duplicate and must be accompanied by a translation into English, in duplicate, of:
- *the whole description*
- *those claims appropriate to the UK (in the language of the proceedings)*

including all drawings, whether or not these contain any textual matter but excluding the front page which contains bibliographic information. The translation must be verified to the satisfaction of the Comptroller as corresponding to the original text.

The Patent Office

Filing of translation of European Patent (UK) under Section 77(6)(a)

Form 54/77 **Patents Act 1977**

❶ European Patent number

1 Please give the European Patent number:
0223809

❷ Proprietor's details

2 Please give the full name(s) and address(es) of the proprietor(s) of the European Patent (UK):

Name Institut Francais De Recherche Scientifique Le Developpment En Cooperation (ORSTOM).

Address 24, rue Bayard,
75008 Paris
France.

Postcode

ADP number (if known):

❸ European Patent Bulletin date

3 Please give the date on which the mention of the grant of the European Patent (UK) was published in the European Patent Bulletin or, if it has not yet been published, the date on which it will be published:

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Date 04 / 02 / 91
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Have you attached:

☒ *one duplicate copy of this form?*

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☐ *any continuation sheets (if appropriate)?*

PATENTS ACT - 1977

AND PATENTS (AMENDMENT) RULES 1987

IN THE MATTER OF a European Patent (UK) in the name of

INSTITUT FRANCAIS DE RECHERCHE POUR LE DEVELOPPEMENT EN COOPERATION - ORSTOM

I, Graham Cross, a translator of 6 Grant Drive, Carlines Park, Ewloe, Clwyd, CH5 3RR, Great Britain

fully conversant with the French and English languages, hereby certify that to the best of my knowledge and belief the following is a true translation into the English language, which has been made/compared by me and for which I accept responsibility, of the specification of the

European Patent No.: 0 223 809

(Application No.: 86 903 425.6)

Signed this 10th day of June 1987

.....

G. CROSS

This invention relates to a method for producing spores of filamentous fungi.

Filamentous fungi are widely used in fields as varied as foodstuff fermentation, the pharmaceutical industry and the production of enzymes for biological molecules by biosynthesis or hemisynthesis.

The spores of filamentous fungi are the resting and reproductive forms of these microorganisms. These spores represent the starting point for all these applications because they can be used as for storage and seeding, and they also act in an extensive way in the process itself either as an inoculum or as the spores themselves in order to effect the bioconversions required.

Various techniques for the production of these spores are known. The oldest, but the most rudimentary, consists of culturing the organism on the surface of a gel medium in either a Petri dish in the laboratory or in Roux flasks for practical applications. Although reliable, because complete asepsis can be ensured, this technique causes serious problems with the harvesting of spores and the handling of a large number of flasks when the quantity required is more than trifling. For this reason this technique can only remain non-industrial and only find application in special cases.

The technique of producing spores on plates involves the culturing of fungi on plant substrates such as wheat bran, straw and various starch-containing products or residues laid in a layer several centimetres thick and placed in incubators. Complex automatic devices for filling and emptying the plates have been proposed. However the product obtained does not consist of pure spores, but a mixture of spores, fungal mycelium and plant residues. Also it is very difficult to maintain aseptic conditions. Spore harvesting, environmental contamination and variability of the organism are major problems.

More recently it has been shown that fungal spores can be produced in liquid cultures using sterilisable fermenters. This is undoubtedly a real advance, but it is only possible under special conditions and for a limited number of fungi. On the other hand the suspension recovered contains not

only spores but also a large proportion of metabolites and all forms of cell debris, which can cause problems. Finally, maintaining conditions for effective aeration in the liquid medium over long incubation periods results in the costly expenditure of energy. In addition to this, among the many fermenter devices which have been proposed for the culture of microorganisms in liquid medium, there are rotary disc fermenters whose principle of operation is based essentially on discs which rotate permanently throughout the incubation period and which are immersed in the liquid nutrient medium and the atmosphere alternately.

This invention relates to a method the producing spores of filamentous fungi characterised in that it involves the implementation of the following successive operations within the rotary disc fermenter:

a) a solidifying agent is added to a culture medium containing at least one growth substrate and culturing agent;
b) this culture medium is placed on a rotary discs in the fermenter;
c) the whole of the fermenter is sterilized;
d) the culture medium is cooled to a temperature remaining above the solidifying point of the said medium;
e) the said culture medium is inoculated with spores of filamentous fungi;
f) the said culture medium is homogenized by rotating the discs of the fermenter slowly;
g) while maintaining a slow rotation of the fermenter discs, the medium is solidified by lowering the temperature of the fermenter abruptly, which has the effect of distributing the medium on the discs;
h) after bringing the rotary discs to a standstill, the inoculated medium is left to incubate while providing a circulation of air in the fermenter with controlled humidity and temperature during the time required for the conidia to mature, and
i) after the spores have developed uniformly on the surface of the fermenter discs, the spores are separated and harvested by sweeping the moving discs in a rapid rotary motion, with the aid of a fluid preferably containing a surfactant and/or sized balls, the mycelial biomass remaining imprisoned in the solidified culture medium which remains fixed on the fermenter discs.

This invention also relates to means required for implementation of the process described above; this consists of a rotary disc fermenter provided with systems for the regulation of temperature and humidity, and a motor which causes the shaft supporting the stack of discs to rotate.

Other features and advantages of this invention will appear from a reading of the following detailed description, particularly in relation to the appended figure which illustrates a particular embodiment of the means required for implementing the method according to the invention.

This new method for production of the spores of filamentous fungi combines the advantage of surface culture on a solid medium, which remains the most reliable and most widespread technique of sporulation, with the use of a special rotary disc fermenter whose principle of use has been fundamentally modified so as to provide a large surface area for sporulation, and in particular so that the spores only can easily be harvested by mere washing of the surfaces, the mycelial biomass remaining trapped within the solid medium. All these operations may be performed in the same sterilisable equipment, ensuring great simplicity and strict asepsis.

The principle of the method is based on the fact that a solidifying agent, for example agar-agar, is added to the nutrient culture medium containing the growth substrate(s) and the culture agents. After sterilisation the medium is cooled and its temperature is maintained above the solidification point, at for example 50°C. The inoculum of spores is then added and the medium is homogenised by slow rotation of the discs.

While the discs are being slowly rotated the atmosphere is suddenly cooled so as to cause the medium to solidify on the surface of the discs.

When the culture medium is distributed over the discs rotation is stopped and the temperature of the device is raised to the incubation temperature. A flow of sterile air, of controlled moisture content and temperature, is maintained throughout the incubation period so as to maintain conditions favourable for growth of the fungus. It should be noted that no mechanical agitation is required during this incubation.

The organism thus develops uniformly throughout the mass of the solidified medium. After 1 to 2 days the free surfaces are covered with a uniform carpet of filaments. Depending on the organism used, sporulation occurs after 2 - 3 days, in a very regular and synchronous manner on all the available surfaces. The spores in the external conidia are thus outside the discs (5), while the vegetative mycelium remains trapped within the solid medium (6). Incubation may be continued for up to 7 to 10 days in order to allow the conidia to mature.

The spores can then be harvested particularly easily by placing a volume of sterile water containing a surfactant, for example TWEEN 80(R) in the fermenter and rotating the discs rapidly for about 10 minutes.

The surfaces of the discs are thereby very efficiently washed, placing the spores in suspension without the mycelial biomass and the majority of the metabolites, which are strongly fixed within the gel medium. Several successive washes may be performed. The spores can then be centrifuged or decanted, washed and dried by evaporation under vacuum, freeze-drying or spraying.

In accordance with another possible method of harvesting sized balls of a diameter less than the distance between the faces of the discs can be introduced in order to encourage dispersion of the spores into the atmosphere. These spores are then harvested dry by rotating the discs together with sufficient sweeping with sterile air to set up turbulence and entraining the spores which are then harvested by a device filtering the air which leaves the fermenter.

When the spores have been harvested the fermenter is washed by introducing a hot detergent liquid which together with rapid agitation causes the medium to melt, sterilises the residual biomass and cleans the device.

In accordance with a preferred method of implementing the process, the reactor described in Figure 1 is of cylindrical shape and is constructed of materials which can withstand sterilisation. The fermenter consists of a cylindrical vessel 1 fitted at one end to a plate 2, fitted with a rotating shaft 3 which supports a stack of rigid discs 4 of a diameter slightly less

than the internal diameter of the vessel. The material, thickness and surface area of the discs may be of any kind provided that they permit satisfactory retention and uniform distribution of the medium during rotation and the solidification stage. These discs 4 may consist of merely one or two steel grids a few mm thick having a mesh of from 2 to 5 mm. The spacing and arrangement of these grids may be variable. In Figure 1 which describes an embodiment of this device the discs consist of two steel grids 2 mm thick spaced 2 mm apart so that after the medium has been distributed and solidified a disc 6 mm thick is obtained. In a preferred embodiment the space in between the gel coated disc is 10 mm.

Forced sweeping of the reactor by a flow of sterile air humidified by bubbling may be provided via an upper inlet (7) and a lower outlet (8) by means of which the reactor can also be filled and emptied. Devices for the monitoring and control of temperature, relative humidity and the composition of the atmosphere in the reactor may prove useful.

A reactor such as just described has been used to implement the invention in the form of a reactor of 1500 cm^3 fitted with 10 plates of 5 mm representing a free surface area of 1270 cm^2.

Tests involved the production of spores from fungi belonging to the genuses Aspergillus, Trichoderma, Penicillium. In actual fact a strain of Aspergillus hennebergii from the A. niger group and a strain of Penicillium isolated in the laboratory were selected to represent the genuses Aspergillus and Penicillium. Two strains from an international collection, Trichoderma harzianum CCM-F-470 and Penicillium camembertii CCM-F-378, were selected to represent the genuses Trichoderma and Penicillium.

In an example of a preferred embodiment of the method the culture medium consisted of one litre of water containing 100 g of manioc flour, 4 g of KH_2PO_4, 8 g of $(NH_4)_2SO_4$, 2 g of urea and 20 g of agar agar. 300 ml of this medium were placed in the reactor which was then sterilised at 120°C for 30 minutes. The device was then cooled and when the temperature of the medium was 50°C the inoculum of spores was introduced in a sterile manner. The medium was then homogenised by rotating the discs at a rate of 60 rpm for a few minutes. After homogenisation a flow of cold sterile air at a

rate of 200 l/h caused the culture medium to solidify on the grids and produce the discs of medium. After 30 minutes rotation was stopped and the fermenter was vented with a flow of 10 litres/h of sterile air saturated with water at the incubation temperature, which may be 30°C, but varies depending on the organism cultured.

After 7 days the spores were harvested by placing 500 ml of sterile water containing a few drops of TWEEN 80 into the fermenter. Aeration was stopped and the disc were rotated at 100 - 200 rpm for 10 minutes. The suspension of spores was then harvested, and the number of spores was calculated by direct counting under a microscope in a Malassez cell after appropriate dilution. Two additional washes using 250 ml of water were performed. The suspension of spores was then decanted, and after the supernatant had been removed a dry powder of spores was obtained by evaporation of the concentrated suspension under vacuum. This spore powder could be used to resuspend the spores easily for use, without interfering surface tension phenomena.

Table I indicates the results obtained for four strains of fungi. It was found that the first wash harvested 75 to 90% of the spores within the device and produced a concentrated spore suspension containing at least 1.1×10^8 to 4.2×10^8 spores per ml.

The quantity of spores formed per cm^2 varies between 3×10^7 and 2.2×10^8 spores/cm^2. The yield of spores by weight in relation to the quantity of substrate placed in the fermenter varies from 5 to 14%, which represents a substantial level of conversion into spores.

In view of the above this invention makes it possible to produce fungal spores under aseptic conditions and by means of a relatively simple technique, in a convenient manner which does not require long and costly manipulations. It also avoids all risks of environmental contamination. Also this invention is characterised in that pure spores are obtained without contamination by other microorganisms and by metabolites or mycelial residues, which are essentially retained within the device at the time of harvesting. The yields obtained are greater than or comparable to those which can be obtained by other conventional techniques which are

difficult to implement. Finally this invention is characterised by the small size of the equipment in relation to the quantities of spores obtained.

TABLE I

	Aspergillus niger (hennebergii)	Trichoderma harzianum	Pencillium sp	Penicillium camembertii
1st wash, 500 ml	75%	76%	87%	92%
2nd wash, 250 ml	15%	24%	11%	6%
3rd wash, 250 ml	10%	5%	2%	2%
Number of spores harvested in 1 litre	7.3×10^{10}	2.8×10^{11}	1.5×10^{11}	3.8×10^{10}
Concentration of spores/ml 1st	1.1×10^{8}	4.2×10^{8}	2.6×10^{8}	6.9×10^{7}
2nd	4.3×10^{7}	2.6×10^{8}	6.6×10^{7}	9.1×10^{6}
3rd	3.0×10^{7}	5.6×10^{7}	1.2×10^{4}	3.0×10^{6}
Number of spores formed per cm^2	5.75×10^{7}	2.2×10^{8}	1.2×10^{8}	3.0×10^{7}
Number of spores formed per g of substrate	2.5×10^{9}	9.3×10^{9}	5.0×10^{9}	1.25×10^{9}
Weight of 1 spore (g)	6.0×10^{-11}	1.3×10^{-11}	1.1×10^{-11}	4.0×10^{-11}
Overall yield by weight in relation to carbon-containing substrate, weight of spores/g	14.6%	12.1%	5.75%	5%

CLAIMS

1. Process for producing spores of filamentous fungi, characterized in that it involves the implementation in a rotary-disc fermenter of the following successive operations:

a) a solidifying agent is added to a culture medium containing at least one growth substrate and culturing agents;

b) this culture medium is placed on the rotary discs in the fermenter;

c) the whole of the fermenter is sterilized;

d) the culture medium is cooled to a temperature remaining above the solidifying point of the said medium;

e) the said culture medium is inoculated with spores of filamentous fungi;

f) the said culture medium is homogenized by rotating the discs of the fermenter slowly;

g) while maintaining a slow rotation of the fermenter discs, the medium is solidified by lowering the temperature of the fermenter abruptly, which has the effect of distributing the medium on the discs;

h) after bringing the rotary discs to a standstill, the inoculated medium is left to incubate while providing a circulation of air in the fermenter with controlled humidity and temperature during the time required for the conidia to mature, and

i) after the spores have developed uniformly on the surface of the fermenter discs, the spores are separated and harvested by sweeping the moving discs in a rapid rotary motion, with the aid of a fluid preferably containing a surfactant and/or sized balls, the mycelial biomass remaining imprisoned in the solidified culture medium which remains fixed on the fermenter discs.

2. Process according to Claim 1, characterized in that it is applied in the production of spores of fungi whereof the type of reproduction is by means of conidia.

3. Process according to one of Claims 1 and 2, characterized in that it is applied in the production of spores of fungi belonging to the species Aspergillus, such as Aspergillus niger, Trichoderma, such as Trichoderma harzianum, or Penicillium such as Penicillium camembertii.

4. Process according to one of Claims 1 to 3, characterized in that the culture agents are in the form of a carbon source such as starch, a nitrogen source such as a mixture of ammonium sulphate and urea, a phosphorus source and other mineral or organic compounds.

5. Process according to one of Claims 1 to 4, characterized in that the solidifying agent added in the course of step a) is agar-agar.

6. Process according to one of Claims 1 to 5, characterized in that the washing fluid used in the course of step i) is sterile water to which a surfactant such as TWEEN 80 has been added.

7. Process according to one of Claims 1 to 6, characterized in that the sized balls used in the course of step i) have a diameter less than the interface distance between the discs.

8. Process according to one of Claims 1 to 7, characterized in that the suspension of harvested spores is concentrated by centrifuging or decanting, washed and then dried by evaporation in vacuo, freeze-drying or spraying.

9. Device for implementing the process according to one of Claims 1 to 8, characterized in that it is in the form of a rotary-disc fermenter provided with systems for regulating the temperature and the humidity, and with a motor enabling the shaft supporting the stacked arrangement of discs to be rotated, at least one surface of these discs being formed by a screen having

a mesh size from 2 to 5 mm.

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LONDON WC1R 5DJ
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24 JAN 2000
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Aktenzeichen/File No./No. du Dossier
RS 103977 GB
Datum/Date
21.01.00

Das Europäische Patentamt übermittelt hiermit den Standardrecherchenbericht zu dem unten bezeichneten Antrag; Kopien der im Recherchenbericht angeführten Schriften werden in)r Anlage beigefügt.

The European Patent Office herewith transmits the Standard Search Report relating to the request indicated below; copies of the documents cited in the search report are enclosed.

L'Office Européen des Brevets à l'honneur de vous transmettre ci-joint le Rapport de Recherche concernant la demande désignée ci-dessous; des copies des documents cités sont jointes.

Zeichen und Datum des Antrages Applicant's reference and date Références et date de la demande	EMR/ACS/5291201
) Dokument, Gegenstand der Recherche Document subject of the search Objet de la recherche	GBA 9902757
Einreichungstag Filing date Date de dépôt	08/02/1999
Beanspruchte Priorität Priority claimed Priorité revendiquée	

OFFICE EUROPÉEN DES BREVETS
Pour le Vice-Président,

European Patent Office

STANDARD SEARCH REPORT

File RS 103977

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim
X	PATENT ABSTRACTS OF JAPAN vol. 015, no. 154 (C-0825), 18 April 1991 (1991-04-18) & JP 03 030665 A (MITSUBISHI HEAVY IND LTD), 8 February 1991 (1991-02-08) * abstract * & JP 03 030665 A (MITSUBISHI HEAVY IND LTD)	1-21
A	FR 403 203 A (LABORATOIRES DE MONTREUX)	

TECHNICAL FIELDS SEARCHED (Int.CL.7)

C12M

The present search report has been drawn up for all claims

Date of completion of the search	Examiner
13 January 2000	Coucke, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C17)

ANNEX TO THE STANDARD SEARCH REPORT NO.

RS 103977

This annex lists the patent family members relating to the patent documents cited in the above-mentioned search report.
The members are as contained in the European Patent Office EDP file on
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13-01-2000

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 03030665 A	08-02-1991	NONE	
FR 403203 A		NONE	

EPO FORM P0469

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
5291299		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00378	08/02/2000	08/02/1999
International Patent Classification (IPC) or national classification and IPC C12M1/26		
Applicant BIODIVERSITY LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30/06/2000	02.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Diez Schlereth, D Telephone No. +49 89 2399 7488

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00378

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-35 as originally filed

Claims, No.:

1-41 as originally filed

Drawings, sheets:

1/8-8/8 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	21,27-32,36
	No:	Claims	1-20,22-26,33-35,37-41
Inventive step (IS)	Yes:	Claims	21
	No:	Claims	1-20,22-41
Industrial applicability (IA)	Yes:	Claims	1-41
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

item V.

1.) Reference is made to the following documents:

D1: EP-A-0 259 116
D2: JP-A-03 030 665 (Patent Abstracts of Japan, vol. 015, No 154, C-0825, 1991)
D3: CH-A-552 063

2.) The subject-matter of claims 1-13, 17-20, 22-26, 33-35 and 37-41 is not novel within the sense of Art. 33 (2) PCT, the reasons being as follows:

D1 discloses a process (and an apparatus) for selectively culturing motile bacteria, which comprises (i) placing a material suspected of containing bacteria into a (first) culture vessel containing liquid nutrient medium (first body of growth supporting material) suitable for bacterial growth, (ii) providing a carrier which supports a solid nutrient medium (second vessel having a second body of growth supporting material) for selective bacterial growth, (iii) dipping partly said carrier into the liquid nutrient medium to allow migration of the motile bacteria from the liquid medium (first location) to the solid carrier (second location), and (iv) culturing further the bacteria to allow bacterial growth within the solid carrier (p. 2, l. 58-63; p. 3, l. 1-40; p. 4, l. 3-4, 21-24, 38-56; p. 5, l. 1-22; p. 8, l. 47-51; p. 9, l. 30-55, 63-65; p. 10, l. 1-5, 41-65; p. 11, l. 1-12; Figs. 2, 5-6, 9 & 11). This document anticipates the subject-matter of claims 1-13, 17-20, 22-26, 33-35 and 37-41.

D2 discloses a device (and a process) for automatic subculturing (see Abstract). It would appear that this document also anticipates the subject-matter of claims 1-13, 17-20, 22, 34 and 37-39.

D3 discloses a process (and a device) for bacterial culture, which is based on (i) placing an ampoule containing an inoculated nutrient growth medium into a flexible tubular membrane which contains a clean nutrient growth medium, (ii) defining different closed segments of the volume enclosed by the membrane by squeezing the membrane with removable clamps, wherein the ampoule is kept in a first closed segment, (iii) breaking the ampoule and letting the microorganisms grow into the first segment in the direction of the second segment. By removing and changing the

position of the clamps the microorganisms are directed to grow within a further closed segment along the whole tubular membrane (col. 1, l. 20-40; Figs. 1-4). This document anticipates the subject-matter of claims 1-13, 17-20, 22, 34 and 37-39.

3.) The subject-matter of independent claims 14 and 15 relates to the use of a (known) microorganism which has been stored following the known process of claims 1-13 in a conventional (known) process for manufacture of a (known) metabolite, and to the use of said (known) metabolite in a (known) method of preparing a (known) pharmaceutical preparation. Therefore, the subject-matter of claims 14 and 15 cannot be considered novel within the sense of Art. 33 (2) PCT.

4.) A pharmaceutical preparation prepared by the method of claim 14 cannot be distinguished from other pharmaceutical preparations known from the prior art, since the chemical nature of the metabolite is not influenced by the process used for its manufacture. Therefore, the subject-matter of independent claim 16 is not considered to be novel within the sense of Art. 33 (2) PCT.

5.) Feeding the non-novel storage device of claim 26 with a vegetable food stuff, a quantity of cereal, seed,pulse, or certain agricultural crop byproducts (claims 27-32) seem to be obvious alternatives for the skilled person, which do not result in any unexpected technical effect. Therefore, the subject-matter of claims 27-32, although formally novel (Art. 33 (2) PCT), is not considered to be inventive within the sense of Art. 33 (3) PCT.

6.) Providing the non-novel storage container with two removable closure means (e.g. two caps) seems to be an obvious constructional choice, which does not result in any unexpected technical effect. Therefore, the subject-matter of dependent claim 36 is considered to be novel (Art. 33 (2) PCT), but not inventive within the sense of Art. 33 (3) PCT.

7.) The subject-matter of dependent claim 21 (a storage device) can be considered novel and inventive within the sense of Art. 33 (2) and (3) PCT for the following reasons:

Providing the storage device of claims 17-20 with a "housing" (32) having "formations"

(34) at the first and second locations of the growth medium, **each "formation" being suitable to engage with a cooperative "formation" of another of said storage device** (claim 21) results in the following technical effect: several "housings" can be serially connected and sealed from the atmosphere by cooperative engagement of said "formations".

A device according to claim 21 is capable of maintain a microorganism of filamentous nature, by repeated subculturing without suffering from the effects of genetic segregation. Furthermore, contamination is limited by excersing caution when connecting and disconnecting housings from each other.

The skilled person equipped with the teaching of D1-D3 would have had neither motivation nor technical guidance for providing the storage device of D1 with "formations" being suitable to engage with a cooperative "formation" of another of said storage device in order to arrive at a device as claimed in claim 21 (see, however, item VIII.1 below).

item VII.

Contrary to the requirements of Rule 5.1 (a) (ii) PCT, the relevant background art disclosed in the documents D1-D3 is not mentioned in the description, nor are these documents identified therein.

item VIII.

1.) The word "formation" does not have a precise meaning for the skilled person and renders the subject-matter of claim 21 unclear (Art. 6 PCT, see PCT Guidelines III-4.2). Furthermore, the terminology used in claims 21 and 41 as regards the features – "housing (32)/ receptacle (32)" and "formations (34)/attachment means (34)" is not consistent with that used in the description ("body (32)" and "**external screw thread (34)**" and renders the subject-matter of said claims unclear (Art. 6, Rule 10.2 PCT, PCT Guidelines II-4.14).

2.) Although claims 17 and 37 have been drafted as separate independent claims, they appear to relate effectively to the same subject-matter and to differ from each other

only with regard to the definition of the subject-matter for which protection is sought and in respect of the terminology used for the features of that subject-matter. The aforementioned claims, therefore, lack conciseness. Moreover, lack of clarity of the claims as a whole arises, since the plurality of independent claims makes it difficult, if not impossible, to determine the matter for which protection is sought, and places an undue burden on others seeking to establish the extent of protection. Hence, these claims do not meet the requirements of Art. 6 PCT.

3.) The difference between "tubular" (claim 18) and "cylindrical" (claim 19), and between "retaining means" (claim 23) and "retaining member" (claim 24) is not clear from the wording of the claims and renders their subject-matter unclear (Art. 6 PCT).

4.) The description is not adapted to the wording of the claims (Art. 6 PCT, PCT Guidelines III-4.3). It would appear that the wording of claims 26-27, 30-32 and 37-41 is not supported by the description.